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Abstract: BACKGROUND AIMS: The organic anion-transporting polypeptide 1B1 (OATP1B1) is an anion exchanger expressed at the hepatocyte sinusoidal membrane, which mediates the uptake of several endogenous metabolites and drugs. OATP1B1 expression level and activity are major sources of inter-patient variability of pharmacokinetics and pharmacodynamics of several drugs. Besides the genotype, factors that contribute to the inter-individual variability in OATP1B1 expression level are practically unknown. The aim of this work was to uncover novel epigenetic mechanisms of OATP1B1 regulation. METHODS: A functional screening strategy to assess the effect of microRNAs on the uptake of estrone-3-sulphate, an OATP1B1 substrate, into human hepatocellular carcinoma (Huh-7) cells was used. microRNA-206 (miR-206) expression in human liver tissues was measured by real-time RT-PCR. OATP1B1 expression in Huh-7 and in human liver tissues was assessed by real-time RT-PCR, Western blotting and immunostaining. The mRNA-miRNA interaction was assessed by reporter assay. RESULTS: miR-206 mimic repressed mRNA and protein expression of OATP1B1 in Huh-7 cells. The intracellular accumulation of estrone-3-sulphate was reduced by 30% in cells overexpressing miR-206. The repressive effect of miR-206 on the activity of the firefly luciferase gene 2 under the control of the OATP1B1 3' untranslated region was lost upon deletion of the predicted miR-206 binding site. Hepatic miR-206 level negatively correlated with OATP1B1 mRNA and protein levels extracted from normal human liver tissues. CONCLUSIONS: miR-206 exerts a suppressive effect on OATP1B1 expression by an epigenetic mechanism. Individuals with high hepatic levels of miR-206 appear to display lower level of OATP1B1.

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microRNA-206 modulates the hepatic expression of the organic anion transporting polypeptide 1B1

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Abbreviations

organic anion transporting polypeptide, OATP; solute organic carrier, SLCO; microRNA-206, miR-206; estrone-3-sulfate, E3S; 3'-untranslated region, 3'UTR.

Conflict of interest statement

Nothing to declare

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Authors' contribution

MV and GAK-U conceptualized the work. TE, BvR, ZG, CH, JV, BS, TvG, MV and GAK-U contributed to perform the research, collect and analyze data. TE and MV wrote the original draft of the manuscript. BvR, ZG, CH, JV, BS, TvG and GAK-U critically revised the manuscript. All authors approved the final version of the article.

ABSTRACT

Background & Aims: The organic anion transporting polypeptide 1B1 (OATP1B1) is an anion exchanger expressed at the hepatocyte sinusoidal membrane, which mediates the uptake of several endogenous metabolites and drugs. OATP1B1 expression level and activity are major sources of inter-patient variability of pharmacokinetics and pharmacodynamics of several drugs. Besides the genotype, factors that contribute to the inter-individual variability in OATP1B1 expression level are practically unknown. The aim of this work was to uncover novel epigenetic mechanisms of OATP1B1 regulation.

Methods: A functional screening strategy to assess the effect of microRNAs on the uptake of estrone-3-sulfate, an OATP1B1 substrate, into human hepatocellular carcinoma (Huh-7) cells was used. microRNA-206 (miR-206) expression in human liver tissues was measured by real time RT-PCR. OATP1B1 expression in Huh-7 and in human liver tissues was assessed by real time RT-PCR, western blotting and immunostaining. The mRNA-miRNA interaction was assessed by reporter assay.

Results: miR-206 mimic repressed mRNA and protein expression of OATP1B1 in Huh-7 cells. The intracellular accumulation of estrone-3-sulfate was reduced by 30% in cells overexpressing miR-206. The repressive effect of miR-206 on the activity of the firefly luciferase gene 2 under the control of the OATP1B1 3' untranslated region was lost upon deletion of the predicted miR-206 binding site. Hepatic miR-206 level negatively correlated with OATP1B1 mRNA and protein levels extracted from normal human liver tissues .

Conclusions: miR-206 exerts a suppressive effect on OATP1B1 expression by an epigenetic mechanism. Individuals with high hepatic levels of miR-206 appears to display lower level of OATP1B1.

Keywords

OATP1B1, microRNA-206, liver, epigenetics

Lay Summary

- The organic anion transporting polypeptide 1B1 (OATP1B1) is involved in the hepatic elimination of several endogenous metabolites and drugs.
- Abnormal OATP1B1 expression levels can affect the pharmacological and toxicological profile of a number of widely prescribed drugs.
- High intrahepatic levels of the small non-coding RNA, microRNA-206, repress OATP1B1 expression.

INTRODUCTION

The organic anion transporting polypeptides (OATPs, *SLCO*) are electroneutral exchangers that mediate the transport of a wide variety of endogenous substrates (e.g. thyroid hormones, bilirubin, bile acids) and are important in the disposition of several drugs and other xenobiotics. Up to now, 12 human OATPs have been identified [1,2], of which the liver-specific OATP1B1, encoded by the *SLCO1B1* gene, is best characterized [3,4]. Loss of function mutations of OATP1B1 (and OATP1B3) have been associated with the Rotor syndrome, a rare, benign disease presenting with conjugated and unconjugated hyperbilirubinemia [5-7]. Knock-out mice for *Slco1b2*, the orthologue of the human *SLCO1B1* and *SLCO1B3* genes, displayed higher level of unconjugated bile acids in plasma, suggesting a key role in clearing the blood from unconjugated bile acids [8].

Data from several pharmacogenetic studies highlight the role of OATP1B1 expression level and activity in the pharmacokinetic/pharmacodynamic inter-patient variability of a number of drugs. A genome-wide study on patients under treatment with the cholesterol-lowering drug simvastatin associated two single nucleotide polymorphisms (SNPs) in the *SLCO1B1* gene with the risk of myopathy: the noncoding rs4363657 and the nonsynonymous rs4149056 (c.T521C, p.V174A), with the latter resulting in impaired protein trafficking and activity *in vitro* [9,10]. Genetic variants of the *SLCO1B1* gene have been associated with a lower clearance of methotrexate and a lower incidence of methotrexate-related mucositis in a genome-wide study on children with acute lymphoblastic leukaemia (ALL) treated with high-dose methotrexate [11]. This finding was replicated in a larger cohort of patients with ALL treated with different regimens of high-dose methotrexate [12]. Similar results have been reported for other statins, the antihistamine fexofenadine and antiretroviral drugs [13-15]. The extensive

genetic evidence for the dominant role of OATP1B1 in drug hepatic clearance and its large substrate specificity led the regulatory agencies to add OATP1B1 (and OATP1B3) as a target of *in vitro* drug-drug interaction prediction [16,17].

The genetic variability in the *SLCO1B1* gene can only explain part of the relatively high inter-individual variability of hepatic OATP1B1 protein expression [18]. The role of non-genetic factors (e.g. age and nuclear transcription factors) in the basal expression level of the OATP1B1 may be marginal [18]. The hepatocyte nuclear factor 1 homeobox A (HNF1α) activates transcription of the *SLCO1B1* gene through direct binding to the promoter region [19]. The farnesoid X receptor (FXR, NR1H4), which is activated by bile acids, has also been shown to regulate transcription of the *SLCO1B1* gene, and is likely to be relevant in cholestatic condition [20,21]. Little is known about the role played by microRNAs in regulating OATP1B1 expression [22,23]. We adopted a functional screening strategy that assessed the effect of microRNAs on the uptake of estrone-3-sulfate (E3S), a prototypic OATP substrate, into Human hepatocellular carcinoma-derived (Huh-7) cells. We found that microRNA-206 (miR-206), by binding to the 3' untranslated region (3' UTR) of the OATP1B1 mRNA, may contribute to the post-transcriptional regulation of OATP1B1. miR-206 is a 22-nucleotide non-coding RNA located in an intergenic region of chromosome 6. miR-206 is considered a myomiR because it is mainly expressed in the skeletal muscle, yet it has also been recently shown to have a role in liver physiology [24-28].

METHODS

Patients and Liver Tissues

All the procedures followed the ethical standards in accordance with the Helsinki declaration and with the local law for medical scientific research with human beings. The local medical ethics committee “Medisch Ethische Toetsings Commissie van het Amsterdam UMC, locatie AMC” approved the protocol for this study before the General Data Protection Regulation (GDPR) law came into force, waiving the need of inform consent because the study employed unused material collected from treatment-purpose hepatectomies. Patients with chronic hepatitis, hepatocellular carcinoma and cholangiocarcinoma were excluded. The analysis was performed on the surrounding normal tissue from patients who underwent partial hepatectomy and with normal serum alpha-fetoprotein. The tissue was defined normal when the following features were present: (i) normal distribution of portal tracts and central veins, (ii) normal architecture of liver cell plates without significant inflammation, cholestasis and/or fibrosis, and copper and iron levels. Steatosis was graded as either negative (0%), less than 5%, 5-33%, 34%-66% and >66%. Inflammation was graded from one to three (mid, moderate, severe), for lobular, interface and portal inflammation. The normal tissue was also assessed for presence of ballooning, bilirubinostasis, siderosis, and copper accumulation. Fibrosis was graded as following: none (0), perisinusoidal or periportal (1), perisinusoidal and portal/periportal (2), bridging fibrosis <50% (3), bridging fibrosis > 50% (4) and cirrhosis (5). More information about patient characteristics is listed in table 1.

Reagents

[6,7-^{3}H(N)]estrone-3-sulfate ([^{3}H]E3S, specific activity 45.6 Ci/mmol) was purchased from PerkinElmer (Boston, MA). Non-labelled E3S was provided by Sigma-Aldrich (St. Louis, MO). Lipofectamine 2000, miRNA mimic 206 (#4464066, miR-206), negative

control (#4464058), the miR-206 (#000510) and the U6 snRNA (#001973) amplification assays were purchased from ThermoFischer Scientific (Waltham, MA). The polyclonal anti-OATP1B1 antibody raised in rabbit was previously generated in our laboratory[29]. The antibody was affinity purified against the peptide used for immunization using a standard protocol. Anti-pan-actin, anti-protein disulphide isomerase (PDI) and horseradish peroxidase-conjugated secondary antibodies were provided by ThermoFischer Scientific (Waltham, MA). Penicillin/streptomycin mixtures, RPMI1640 and DMEM culture media were purchased from ThermoFischer Scientific (Waltham, MA). Biowest fetal bovine serum (FBS) was provided by VWR (Dietikon, CH).

Cell lines

Human hepatocellular carcinoma-derived (Huh-7) cells and Chinese Hamster Ovary (CHO) cells were grown at 37 °C with 5% CO_2 and 95% humidity, in RPMI1640 and DMEM medium, respectively. Both media were supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. DMEM medium was enriched with 100 µg/ml L-proline. CHO cells stably transfected with the open reading frame of OATP1B1 (1B1-CHO) were maintained under the selective pressure of G418 Geneticin at the extracellular concentration of 400 µg/ml [30,31].

In silico prediction

The open access softwares MiRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/, University of Heidelberg; Germany), Targetscan 7.0 (http://www.targetscan.org/vert_70/, Whitehead Institute for Biomedical Research,

Cambridge, MA) and DIANA tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php, University of Thessaly, Greece) were employed to identify possible binding sites of the mature miR-206 and the OATP1B1 mRNA.

Cloning of the full-length and the truncated 3' UTR of the OATP1B1 mRNA

The sequence of the complete (605 bp) and the truncated (r.2762_2786del, 582 bp) 3'-untranslated region (3'UTR) of the *SLCO1B1* gene (Accession NG_011745) were amplified from 100 ng of Human Genomic DNA (ThermoFischer Scientific, Waltham, MA), blunt ligated in pGEM-T vector and then subcloned in pmirGLO. Primer sequences used for the cloning of the complete OATP1B1 3'UTR were: 5' -TCTAGAGGGGAGAAAAAAAGCCACTTCTG- 3' (Fw) and 5' -GCTAGCTTGCAAATATTGGAATGTCTTTATTTCTTC– 3' (Re). Primer sequences used for the cloning of the truncated OATP1B1 3'UTR were: 5' -GCTAGCGGGGAG AAAAAAAGCCACTTCT- 3' (Fw) and 5' -TCTAGATCTTCCACAATACTTAAATGT ATT- 3' (Re). XbaI and NheI were used to subclone into the pmirGLO reporter plasmid. The final constructs were digested and resolved on a 0.8% agarose gel. The inserts were then sequenced by Microsynth (Balgach, Switzerland).

RNA extraction and real time reverse transcription RT-PCR

Total RNA from cells or from frozen tissue samples was extracted using the TRIzol Reagent (Life Technologies, Carlsbad, Ca). mRNAs were reverse transcribed to cDNA using random hexamers as primers and MultiScribe Reverse Transcriptase (Life Technologies, Carlsbad, Ca). For miR-206 and U6 level assessment, 10 ng of total RNA were reversed transcribed according to the manufacturer's instructions, using

specific primers commercially available. The cDNA products were used as template for PCR amplification by Taqman® assay analysis with the TaqMan Fast Advanced Master Mix (ThermoFischer Scientific, Waltham, MA) on ABI 7900HT (AB Applied Biosystems, Foster City, CA, USA).

Immunofluorescence

Huh-7 cells were seeded on chamber-slides at a density of $5x10^4$ cells/well (LAB-TEK, Naperville, IL). Twenty-four hours later, cells were transfected with miR-206 mimic or negative control. Seventy-two hours post-transfection, cells were washed with cold PBS, fixed in 4% paraformaldehyde, and permeabilized for 15 min with 0.1% Triton X-100 and 30 min with 0.1% Tween 20/1% BSA in PBS. Then, the cells were incubated at 4 °C overnight with the polyclonal anti-OATP1B1 antibody. After washing, the cells were stained with AlexaFluor 488 goat anti-rabbit IgG (ThermoFischer Scientific, Waltham, MA). Cells were mounted with DAPI (Vector Laboratories) and visualized under a confocal microscope. Immunostaining of OATP1B1 in WT-CHO and 1B1-CHO was performed following the same procedure, and served as negative and positive controls, respectively.

Histological analysis

For diagnostic assessment of liver tissue, the following standard staining protocols were used: Hematoxylin and eosin (HE), Periodic acid–Schiff (PAS), PAS stain in combination with diastase (PAS-D), reticulin (RET), sirius red, elastica van Gieson, copper, iron and Cytokeration 7 (CK7). Immunostaining of OATP1B1 was performed on paraffin sections using a microwave-based antigen-retrieval technique. Sections

were treated with the Envision+ DAB kit (Dako, Denmark) according to the manufacturer's instruction then probed with the anti-OATP1B1. Two unbiased observer assessed the OATP1B1 expression level according to the H scoring system [32]. Three high-power fields were analysed per sample.

Protein sample preparation for Western blotting

Western blotting from liver samples was performed using total membrane fractions isolated from human liver tissues or Huh-7 cells as previously described [33]. For tissue total membrane preparation, at least 100 mg of frozen tissue were homogenized with a Polytron in 300 mM sucrose buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). For Huh-7 cells total membrane preparation, cells were transfected with miR-206 mimic or negative control and 72h post-transfection homogenized with a Polytron in 250 mM sucrose buffer supplemented with protease inhibitor cocktail. Tissue and cell homogenates were spun down at 1300gav in a Sorvall SS34 rotor for 10 minutes. The supernatant was further centrifuged for 1 hour at 100000gav in a Kontron Ultracentrifuge. The pellets containing the liver or the Huh-7 cells total membrane fractions were resuspended in 300 and 250 mM sucrose respectively, aliquoted and stored until use at – 80 °C.

Western blotting

One to two hundred µg of protein samples were resolved on a 8% (w/v) polyacrylamide gel and electroblotted onto nitrocellulose membranes (GE HealthCare, Piscataway, NJ). The membranes were blocked with 5% nonfat dry milk in PBS supplemented with 0.1% (v/v) Tween 20 (PBS-T) and incubated overnight at 4°C with anti-OATP1B1

antibody, followed by probing with horseradish peroxidase-conjugated anti-rabbit IgG antibody. Blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA). The images were acquired and analysed with the Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). As a loading control, the sample blots were stripped and reprobed with anti-actin or anti-PDI followed by horseradish peroxidase-conjugated anti-mouse IgG antibody.

Transport assay

Huh-7 cells were seeded on a 96-well plate at a density of $4x10^4$ cells/well. Twenty-four hours later, cells were transfected with miR-206 mimic or negative control and grown for 72 hours. Uptake of estrone-3-sulfate (E3S) was measured as follows: cells were washed in transport buffer (136 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO4, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 11 mM D-glucose and 10 mM Hepes/Tris, pH 7.4) at 37°C, then incubated for 10 minutes with pre-warmed transport buffer containing E3S at the extracellular concentration of 1 µM, spiked with [^{3}H]E3S. The plate was rapidly transferred on ice and extensively washed with ice-cold transport buffer. Cells were solubilized in 0.1 mL of 1% (w/v) Triton X-100. Seventy-five µl of the lysate was mixed with 3 ml of Scintillation Liquid (Ultima Gold, PerkinElmer, Switzerland) and assessed for intracellular radioactivity by liquid scintillation counting. Protein content was determined by the bicinchoninic acid protein assay on the remaining 25 µl (Interchim, Montluçon Cedex, France).

Luciferase assay

The direct binding of miR-206 to the OATP1B1 3′UTR was assessed by sub-cloning the full or the truncated (r.2762_2786del) OATP1B1 3′UTR into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) downstream of the coding sequences of the firefly luciferase 2 (*luc2*) gene. Huh-7 cells were seeded in 48-well plates at a density of 4×10^4 cells/well. After 24h, the cells were transfected with 100 ng/well of pmirGLO plasmid containing the full or the truncated OATP1B1 3′UTR. The empty pmirGLO plasmid was used as a control. Afterwards, cells were transfected with 0.25 μM miR-206 mimic or the same concentration of an unrelated microRNA used as a negative control. Twenty-four hours later the cells were processed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The luminescent signals were measured with the GloMax Multi Detection System (Promega, Madison, WI).

Statistical Analysis

Statistical comparisons were performed from at least three independent measurements, with the two-tailed Student's t-test. The correlation between OATP1B1 expression and miR-206 levels was assessed by Pearson’s and Spearman's Rank-Order correlation. All statistical analyses were performed using GraphPad Prism (version 5.0 for Windows, GraphPad Software).

RESULTS

Effect of miR-206 on OATP1B1 expression level

Figure 1 shows that the transient transfection of Huh-7 cells with the miR-206 mimic at extracellular concentrations ≥ of 0.25 µM resulted in a 70% reduction of OATP1B1 mRNA level in comparison with that in cells transfected with an unrelated microRNA used as a negative control. To test the impact of miR-206 on the protein level of OATP1B1, Western blotting and immunostaining of OATP1B1 was performed on Huh-7 cells 72h after transfection with the miR-206 mimic or the negative control, at an extracellular concentration of 0.25 µM. The antibody specificity was confirmed on CHO cells stably transfected with OATP1B1 and the respective wild-type, which does not express OATP1B1. Figure 1B shows that OATP1B1 localizes predominantly at the plasma membrane of Huh-7 cells and a weaker staining intensity was observed in cells overexpressing miR-206 as compared with the mock transfected cells (Fig. 1B, bottom panels). Figure 1C shows that OATP1B1 was detected at two different molecular weights (70 kDa and 100 kDa) in 1B1-CHO cells, which are consistent with two different glycosylation patterns[34]. In Huh-7 cells, OATP1B1 appears to be expressed primarily at lower molecular weight (70 kDa) and its expression was reduced by ~ 50% (P=0.01) after transfection with miR-206 mimic (Fig. 1D).

Effect of miR-206 on the intracellular accumulation of estrone-3-sulfate

Estrone-3-sulfate requires a facilitative transport system to enter cells and represents a prototypic substrate of the OATPs. Indeed the uptake of E3S was temperature-dependent and saturated upon co-incubation with an excess of non-labeled E3S, indicating that the uptake of E3S in Huh-7 cells was carrier mediated (Fig. 2A). Huh-7 cells transiently transfected with miR-206 displayed a ~30% lower intracellular level of E3S as compared with the control transfected cells (P=0.003), compatible with a reduction of the OATP1B1 expression level (Fig 2B).

335

336 Impact of the r.2762_2786del in the OATP1B1 mRNA on miR-206-mediated
337 repression of OATP1B1 expression level

338 miR-206 was predicted, by the MiRWalk 2.0 and the Targetscan 7.0 softwares, to bind
339 to the mRNA of the *SLCO1B1* gene in the 3' UTR in close proximity to the polyA tail (r.
340 2771_2778). To assess the direct binding of the miR-206 to the OATP1B1 mRNA, the
341 3' UTR of the OATP1B1 mRNA containing the predicted binding site for the miR-206
342 and a truncated version with a deletion of the last 25 bases were cloned into the
343 pmirGLO vector. The activity of the firefly luciferase was monitored after transient
344 transfection with the miR-206 mimic or an unrelated microRNA as a negative control.
345 In figure 3A it can be seen that the reporter activity was reduced by ~ 60% by the miR-
346 206 mimic as compared with the negative control (P=0.003). The effect of miR-206 on
347 reporter activity was abolished by the r.2762_2786 deletion (P=NS) (Fig. 3B),
348 suggesting that the repressive effect of miR-206 on the OATP1B1 mRNA and protein
349 level was the result of direct binding of miR-206 to the 3' UTR of the OATP1B1 mRNA.

350

351 OATP1B1 and miR-206 expression level in human liver

352 The relative expression levels of OATP1B1 and miR-206 were assessed from the
353 normal liver tissue of twenty patients who underwent partial hepatectomy. All the
354 tissues included in the analysis showed normal distribution of portal tracts and central
355 veins, and normal architecture of liver cells with minor or no signs of inflammation,
356 fibrosis, and copper and iron levels. Seven out of 20 patients were diagnosed with
357 steatosis (table 1). Figure 4 shows that the levels of OATP1B1 mRNA (Fig. 4A) and
358 miR-206 (Fig. 4B) varied substantially among the samples. Notably, individuals with

high miR-206 levels exhibited low levels of OATP1B1 mRNA. Conversely, high mRNA levels of OATP1B1 were associated with low levels of miR-206. A relatively strong negative correlation was found between OATP1B1 mRNA and miR-206 levels (Fig. 4C). The inset in figure 4C describes the level of OATP1B1 mRNA as a function of that of miR-206 when the three patients with the highest miR-206 value were excluded from the analysis. There was no difference in the OATP1B1 mRNA levels between steatotic and non-steatotic livers (Fig. 4D). Interestingly, the patients with the highest miR-206 levels clustered in the non-steatotic group (Fig. 4E). This is in line with the reported protective role of miR-206 in liver steatosis in animals [25,28].

Figure 5A shows representative high- and a low-intensity immunostaining for OATP1B1. Upon relative quantification of 16 out of 20 samples, based on the median value, a low and a high expression groups were defined. It can be seen that patients with the highest miR-206 level clustered together in the low OATP1B1 expression group (Fig. 5A, right panel). For 14 out of 20 samples, enough material was available to assess the protein level of OATP1B1 also by western blotting (Fig. 5B). There was a positive correlation between the mRNA and the protein levels of OATP1B1 (Fig. 5C), and a negative correlation between miR-206 level and the OATP1B1 protein level (Fig. 5D).

DISCUSSION

miR-206 directly interacts with the OATP1B1 mRNA at the level of the 3'UTR, and reduces the mRNA and protein levels, presumably as a consequence of enhanced degradation of the OATP1B1 transcript and/or repression of protein synthesis [35,36].

While the reduction of the OATP1B1 mRNA level by the miR-206 mimic was already achieved at the lowest extracellular concentrations of miR-206 mimic tested (0.05-0.1 μM), the effect was best reproduced at an extracellular concentration of 0.25 μM or more, due perhaps to variability in transfection efficiency. The transient transfection of miRNA mimic at such relatively high concentrations carries the risk of altering gene expression in a non-specific manner [37]. Nevertheless, the effect of the miR-206 mimic on the OATP1B1 expression level in the Huh-7 cells appears to be specific: (i) the transient transfection with the same concentration of an unrelated miRNA mimic (negative control) did not repress OATP1B1 expression; (ii) the miR-206 mimic did not interact with the OATP1B1 3'UTR lacking the miR-206 predicted binding site. This is in line with the observation that most of the transfected miRNA mimic may be retained within vesicles and not accessible for loading into Argonaut as functionally active miRNA [38].

The effect of miR-206 on OATP1B1 expression level *in vitro* was supported by the subsequent observation that individuals expressing high hepatic levels of miR-206 displayed relatively low levels of OATP1B1. There was a negative correlation between the intrahepatic level of miR-206 and both OATP1B1 mRNA and protein levels. In line with a previous report, there was a positive correlation between mRNA and protein level of OATP1B1[18]. Taken together, the data suggest that, especially when expressed at higher levels, miR-206 may affect OATP1B1 expression *in vivo*. Noteworthy, miR-206 may also contribute to the previously reported FXR-mediated indirect repression of OATP1B1 expression [20]. It has been shown that the nuclear receptor SHP (NROB2), a genuine target of FXR, induces miR-206 expression [39].

miR-206 seems to play a key role in the regulation of whole body glucose homeostasis in animals fed a high-fat diet by modulating the expression of glucose-6-phosphate

dehydrogenase, glucokinase and glucose transporters in the intestine and pancreatic islets but not in the liver [40]. Liver-specific overexpression of miR-206 has been shown recently to protect against steatosis by inhibiting the Sterol Regulatory Element Binding Protein 1c (Srebp1c) and the Liver X receptor alpha (LXRalpha) transcriptional activity and by facilitating insulin signalling via insulin receptor phosphorylation [25,28]. Additionally, the level of miR-206 in hepatocellular carcinoma has been found to be lower than that in the normal liver, and miR-206 mimic injection was able to suppress carcinogenesis in two different mouse models of hepatocellular carcinoma [27]. The present work consolidates the potential role of miR-206 in liver physiology. miR-206 mainly originates from skeletal muscle and brown adipose tissue [24,26]. While it cannot be excluded that the miR-206 measured in the present study was synthesized intrahepatically by resident cells (e.g. hepatocytes), it is possible that circulating miR-206 may have entered and accumulated in the liver to regulate hepatocyte function. It was shown, first in plants and then in animals, that microRNAs synthesized in a distant tissue can be delivered systemically, retaining their activity and regulating the expression pattern of the target cell type [41,42].

Although the repressive effect of miR-206 mimic transfection on OATP1B1 mRNA was pronounced, the reduction in transport activity was only moderate, albeit significant. This is not surprising since estrone-3-sulfate, like most OATP1B1 substrates, is also transported by other organic anion transporters, which may not be affected by the transfection with the miR-206 mimic[1]. Likewise, it cannot be ruled out that the reduced uptake of E3S in Huh-7 cells overexpressing miR-206 stems from the repression of some endogenous transporters, besides OATP1B1, that may contribute to the uptake of E3S in these cells. Nonetheless, because OATP1B1 is critical for the rate of elimination of several endogenous compounds and drugs, it is conceivable that the

433 effect of miR-206 levels on the pharmacokinetics of OATP1B1 substrates could be
434 larger than that observed in Huh-7 cells [11,13-15,43].

435 miRNAs are appealing as a class of therapeutics: rather than intercepting a single
436 target as is the case of selective protein inhibitors, miRNAs would modulate entire gene
437 cascades. miR-206 is a promising drug target: its use is under preclinical development
438 in muscle-related diseases such as Duchenne muscular dystrophy and in a number of
439 cancers including rhabdomyosarcoma [44,45]. Our data suggest that the clinical
440 development of miR-206 mimic for injection or of drugs that induces the endogenous
441 miR-206 expression should include extensive drug-drug interaction studies, as miR-
442 206 may confer alterations in the pharmacokinetics and pharmacodynamics of
443 OATP1B1 substrates by repressing OATP1B1 expression and, in turn, activity.

444 To conclude, high hepatic levels of miR-206 may repress the expression of OATP1B1
445 and may explain, at least part of the still unexplained inter-individual variability in
446 OATP1B1 expression and, in turn, of the variable pharmacokinetics and toxicokinetics
447 of many OATP1B1 substrates. Studies on larger cohorts of patients are necessary to
448 establish whether the circulating miR-206 represents a candidate marker to identify
449 patients with lower hepatic clearance of OATP1B1 substrates such as statins and
450 methotrexate.

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Table 1. Patient clinical characteristics.

Parameters	Category	N (%)
Age at diagnosis	< 50	15 (75)
	≥ 50	5 (25)
Gender	M	0 (0)
	F	20 (100)
Histopathological diagnosis	FNH	8 (31)
	HCA	9 (50)
	other	3 (19)
Cholestasis	Yes	1 (6)
	No	19 (94)
Bilirubinostasis	Yes	0 (0)
	No	20 (100)
Siderosis	Yes	0 (0)
	No	20 (100)
Copper traces	Yes	3 (15)
	No	17 (85)
Ballooning	Yes	0 (0)
	No	20 (100)
Steatosis	0	13 (65)
	1	4 (20)
	2	3 (15)
	3	0 (0)
Inflammation	0-1	20 (100)
	2-3	0 (0)
Fibrosis	0-1	20 (100)
	2-3	0 (0)
	4-5	0 (0)

Other diagnoses: one patient with chronic cholestasis secondary to bile duct injury, one patient with liver metastasis of colon carcinoma and one patient with B-cell lymphoma. For steatosis grading: <5% (0), 5-33% (1), 34%-66% (2) and >66% (3). For inflammation grading: none (0), little (1), moderate (2) and severe (3). For fibrosis grading: none (0), perisinusoidal or periportal (1), perisinusoidal and portal/periportal (2), bridging fibrosis <50% (3), bridging fibrosis > 50% (4) and cirrhosis (5).

FIGURE LEGENDS

Figure 1. OATP1B1 expression level in Huh-7 cells overexpressing miR-206. The mRNA and protein level of OATP1B1 in Huh-7 cells were assessed 48h and 72h after transient transfection with miR-206 mimic or the negative control, respectively. Ubiquitin C (UBC) gene and protein disulphide isomerase (PDI) were used as an internal control for mRNA quantification and immunoblotting. mRNA data are expressed relative to that in the control and are the mean ± S.D. of 3 independent experiments (A). CHO cells stably transfected with OATP1B1 were used as a western blotting and immunostaining control. Western blotting and Immunofluorescence images (bar=50 μm) are representative of four independent experiments (B, C). OATP1B1 bands were quantified and expressed relative to the PDI band intensity (D).

Figure 2. Uptake of estrone-3-sulfate in Huh-7 cells. Ten minutes uptake of estrone-3-sulfate at an extracellular concentration of 1 μM in non-transfected cells under the indicated conditions (A) and in cells after 72h transfection with miR-206 mimic or a negative control (B). Data are expressed as the mean ± S.D. of four independent experiments.

Figure 3. OATP1B1 3'UTR activity. Huh-7 cells were co-transfected with the miR-206 mimic or the negative control and the pmirGLO-1B1 3'UTR (A) or the pmirGLO-1B1 3'UTR_r.2762_2786del (B) constructs. Renilla reporter activity was used as an internal control. Values were normalized for those in the empty-vector transfected cells and are expressed relative to that in the control. Data are the mean ± S.D. of four independent experiments.

Figure 4. OATP1B1 mRNA and miR-206 levels in human liver samples. OATP1B1 values are normalized to that of the Ubiquitin C (UBC) (A, C). miR-206 values are

normalized to those of the short RNA U6 (B, D). OATP1B1 mRNA level as a function of the miR-206 level (C). The inset shows OATP1B1 mRNA levels as a function of the miR-206 level upon exclusion of miR-206 value outliers. Data were correlated by Pearson's and Spearman's Rank-Order analysis. OATP1B1 mRNA (D) and miR-206 (E) levels in the steatosis subgroup.

Figure 5. OATP1B1 protein level in human liver samples. Representative OATP1B1 immunohistochemistry of a high and low expressing tissue, and staining intensity quantification. Low expression and high expression samples were clustered based on the calculated median value (A). Western blotting of OATP1B1 from liver total membrane fraction. Pan-actin was used as a loading control (B). OATP1B1 mRNA level as a function of the OATP1B1 protein level (C). OATP1B1 protein level as a function of miR-206 level (D). Data were correlated by Pearson's and Spearman's Rank-Order analysis.